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Detection and Sequence Analysis of the Major Immediate Early and PP150 Gene of Latent Human Cytomegalovirus in Spleen, Liver, and Kidney Tissues of Trauma Victims

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The presence of human cytomegalovirus (HCMV) DNA in liver, spleen, and kidney samples of HCMV-seropositive trauma victims during latency was demonstrated by polymerase chain reaction (PCR), using primers reactive with the major immediate early gene exon 4 and the structural gene pp150. Sequence analysis of the PCR amplificates showed more than 95% homology with the reference HCMV strain AD169. The few mutations observed were mostly distributed randomly. In one subject two types of the MIE-4 gene were detected, and in another subject two types of the pp150 gene were found, suggesting that different strains of HCMV can be found in organs of the same patient during latency.

KEY WORDS: HCMV, latency, PCR

INTRODUCTION

In general, human cytomegalovirus (HCMV), a member of the Herpesviridae family, infects immunocompetent individuals without causing clinical disease [Ho, 1992]. The prevalence of antibodies to HCMV varies from 40 to 80%, depending on the person's socioeconomic status [Yow et al., 1988]. After primary infection, the virus becomes latent but can be reactivated during periods of immunosuppression and this can lead to life-threatening disease [Ho, 1982]. The cell type or types that act as a reservoir for the latent HCMV, however, remain(s) obscure. Polymerase chain reaction (PCR) analysis has shown that both peripheral leukocytes [Stanier et al., 1989; Bevan et al., 1991; Taylor-Wiedeman et al., 1991, 1993] and vascular smooth muscle cells [Melnick et al., 1983; Yamashiroya et al., 1988; Hendrix et al., 1990, 1991] can harbour latent HCMV. The data concerning leukocytes are difficult to interpret because several reports demonstrate that HCMV DNA is present not only in seropositive healthy individuals, but also in those who are seronegative [Stanier et al., 1989; Bevan et al., 1991; Taylor-Wiedeman et al., 1991, 1993], whereas in other reports no HCMV DNA could be detected [Bitch et al., 1992]. There were great differences in DNA preparation procedures and PCR reaction conditions between these reports, which may contribute to the differences observed. Furthermore, in none of these reports were the sequences of the PCR product analyzed, identity only being confirmed by hybridisation with end-labelled oligonucleotides as probes. Significant sequence variation, as described for clinical HCMV isolates within the gB gene [Zhang et al., 1995; Chou and Dennison, 1991], the LS junction [Fries et al., 1994], and the major immediate early gene [UL123EX4; Zaia et al., 1990], would therefore go undetected. Sequence analysis can help control an inherent problem of PCR, which is its susceptibility to artifacts caused by mispriming with genomic DNA, or with DNA from other Herpesviridae. However, PCR is a powerful tool for elucidating epidemiological patterns in the pathogenesis of HCMV [Zaia et al., 1990; Chou and Dennison, 1991; Zhang et al., 1995].

Blood and/or blood products are mostly depleted of leukocytes, and therefore not a major route of HCMV transmission [Chou, 1992], but organ transplantation [Ho, 1982], especially from seropositive donors to seronegative recipients, is a potential route of infection. It therefore seems prudent not only to examine organs available for transplantation such as the liver and kidney, but also other available organs such as the spleen, for the presence of latent HCMV, employing the same techniques used for leukocyte analysis.

In this study, we describe the presence of HCMV nucleic acids, as detected by nested PCR in specimens of spleen, kidney, and liver obtained from previously healthy trauma victims. We also present sequence data

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for the PCR products, proving that it is indeed HCMV which is detected.

MATERIALS AND METHODS

Tissue Specimens

Specimens of spleen, kidney, and liver from previously healthy trauma victims were collected at autopsy, using strict aseptic techniques. All specimens were kept apart and were directly placed, in the autopsy room, in 70% ethanol-phosphate-buffered saline and stored at 4°C until use. Autopsy was performed within 12 hr of death. None of the trauma victims had received blood and/or blood products. Eight patients who died of major brain trauma (two females, six males) provided seven spleen samples, eight kidney samples, and six liver samples. The mean age of the subjects was 38 years (range 18–45 years).

Serology

Seropositivity for HCMV was determined for all subjects, using a latex agglutination test (CMV-scan, Becton & Dickinson, Great Britain) [Verdonck et al., 1987] and an ELISA (Organon-Teknika, The Netherlands). [Kraat et al., 1992] All sera were collected directly on arrival of the subjects at the hospital.

DNA Extraction From Tissues

DNA for PCR analysis was extracted from the ethanol-preserved tissues. Small tissue samples were collected in 1.5 ml polypropylene microcentrifuge tubes. Proteolytic digestion of all samples was carried out overnight at 50°C in a mixture of 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 250 μg/ml proteinase K (Boehringer Mannheim, Germany), and 10 mg/ml sodium dodecylsulphate under constant agitation. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and was finally dissolved in 50 μl distilled water. The DNA was quantified spectrophotometrically. To evaluate the possibility of cross-contamination with HCMV DNA during this procedure, every second sample served as a negative control containing herring sperm DNA only and was processed in the same way as the tissue samples.

Oligomer Synthesis

Oligomers were purchased from Eurogentec (Liege, Belgium). One nested set was derived from the HCMV major immediate early gene exon 4 (UL123EX4). The nucleotide sequences of the primers used were: first reaction: A1 5′-ACGAA-TTCGT-AATGA-AGCGC-CGCAT-TGA-3′ and A2 5′-ACGAG-CTGCA-ATAGT-GACGT-GGGAT-CCA-3′; nested reaction: B1 5′-ACGAT-TTCTC-TGCAT-GAAGG-TCTTT-GC-3′ and B2 5′-ACGAG-CTCGT-GGGAT-CCATA-ACAGT-AA-3′. The nested PCR product had a length of 468 base pairs (bp). A second nested set was derived from the large structural phosphoprotein pp150 [ppUL32 (bpp)] [Akrigg et al., 1986]. The nucleotide sequences of the primers used were: first reaction: C1 5′-GGTGC-TGTTC-AACGA-GCTCA-3′ and C2 5′-GGTCC-AGGTT-AAGGT-CGAGC-3′; nested reaction: D1 5′-TTGGG-ATACT-ACCGC-GAGCT-3′ and D2 5′-TCACG-CAGAA-CATGT-TACGG-3′. The nested product had a length of 360 bp. No cloning experiments or other tests had been performed in our laboratory with DNA coding for pp150 prior to these PCR experiments. All primers were tested for reactivity with DNA sequences stored in the EMBL gene bank database and were demonstrated to match stored HCMV sequences only.

Nested PCR

All PCR experiments were carried out under identical reaction conditions. Primer sets were used in separate reactions, but also in a duplex set-up. The first reaction was performed in 50 μl under standard conditions [Hendrix et al., 1990]. After 30 cycles (melting: 1 min at 94°C; annealing: 1 min at 58°C; and primer extension: 2 min at 72°C) performed on a Perkin-Elmer-Cetus thermal cycler (Perkin-Elmer-Cetus, USA), 1 μl of the reaction mixture was transferred to the second reaction tube containing the nested primer set under reaction conditions identical to those in the first reaction. After 40 cycles, 10 μl of the second reaction mixture was subjected to electrophoresis on 2% agarose gels (Seakem GTG, FMC Bioproducts, USA). Amplificates of both MIE-4 and pp150 target genes with the appropriate length were cloned in vector pUC19 digested with Hind II. One clone containing an amplificon as the insert was subjected to sequence analysis. To analyse the quality of DNA, samples were spiked with 1ↄ gram of plasmid pES [Hendrix et al., 1990] and subjected to PCR.

Sequence Analysis

Initially, sequence analysis was undertaken with a single clone of each tissue sample, using the dideoxy-chain-termination kit as described by the manufacturer (Pharmacia, Uppsala, Sweden). Five of the single clones that demonstrated sequence variation as compared to the HCMV reference strain AD169 were analysed further. For this purpose, a 400 bp fragment of the MIE-4 gene and a 341 bp fragment of the pp150 gene were analysed.

Fidelity Considerations

To investigate the possibility of misincorporation of nucleotides during PCR, due to misreading by the polymerase, 18 separate tissue samples were spiked with purified DNA derived from human embryonic fibroblasts infected with the HCMV reference strain AD169. Nested PCR, cloning, and sequence analysis were carried out as for clinical samples with both nested primer sets.

Prevention of False-Positive-Results

Pre- and post-PCR handling of samples and buffer preparation were done in different laboratories in order to prevent carry-over of reaction products.

TABLE I. Sequence Analysis of Six Clones Obtained From Tissue Samples Showing Sequence Variation in the Initial Clone Tested*

Tissue	MIE-4 mutations	pp150 mutations
Subject 1		
Spleen	None	5x AD169 • 321 (T)
Kidney	3x AD169 • 135 (G) • 257 (T) • 306 (A)	5x AD169 • 211 ( ) 235 (G)
Liver	5x AD169 • 175 (T) 362 (G) 363 (G) 384 (A)	2x AD169 • 59 (G) 140 (C) • 227 (A) 106 (T) • 318 (A) 281 (A) 211 (A) 21 (dG) 26 (C)
Subject 2		
Spleen	None	5x AD169 • 113 (A)
Subject 5		
Spleen	4x AD169 • 176 (G) • 212 (C)	None
Kidney	• 263 (C) 332 (A) 368 (A) 389(A) • 360 (G) 332 (A) 368 (A) 389 (A) • 94 (T) 130 (A) 141 (C) • 211 (G) 263 (C) 332 (A) 360 (G) 368 (A) 389 (A) • 170 (G) 180 (A) • 234 (C)	5x AD169 • 231 (T)
Liver	5x AD169 • 275 (G)	4x AD169 • 157 (G) • 168 (A)
Subject 6		
Kidney	None	5x AD169 • 230 (T)
Liver	None	5x AD169 • 68 (A)
Subject 7		
Spleen	None	5x AD169 • 16 (A) 21 (T)
Kidney	None	None

*None means that in the initial clone no variation in the sequences were detectable.

RESULTS

Serology

All subjects had antibodies against HCMV, as detected by the latex agglutination assay and the ELISA. There was 100% agreement between both tests.

Nested PCR Reaction

All tissue samples produced HCMV-specific reaction products as demonstrated by agarose gel electrophoresis. Samples were positive when tested with MIE-4 or pp150 primers in separate assays, but also when tested in a duplex-PCR set-up. To test for carry-over of reaction products, every second sample serving as a negative control containing only herring sperm DNA was also amplified, but none of these controls demonstrated PCR reactivity.

Fidelity Considerations

Of the tissue samples spiked with HCMV reference strain AD169-infected fibroblast-derived DNA, a total of 16 independent amplificons of the MIE-4 gene were cloned and sequenced. Of these 16 cloned PCR products, 2 showed one mutation and 2 showed two mutations, all randomly distributed. Thus, 1 in 1,066 nucleotides was misincorporated. Sixteen cloned PCR products of the pp150 gene were analysed the same way. There were six mutations in six of the amplificons, giving a misreading rate of 1 in 909 nucleotides.

Sequence Analysis

Most of the cloned PCR products derived from the tissue samples had the same sequence as the reference HCMV reference strain AD169. However, 14 of 44 did

show some mutations (Table I). From these variant samples, but also from randomly chosen samples without mutations, five additional clones were sequenced. Five additional clones of two tissue samples without mutations in the MIE-4 gene, being the spleen of patient 1 and the liver of patient 6, remained without mutations as did additional samples without mutations in the pp150 gene, being the spleen of patient 5 and the liver of patient 7. Only cloned PCR products obtained from the kidney of patient 5 consequently demonstrated mutations at positions 263, 332, 368, and 389 of the MIE-4 gene. Clones from other organs from the same patient did not show these variations. Within the pp150 gene, the same mutation at position 211 was observed in one clone from the kidney and in one clone from the liver from patient 1. The other mutations were located randomly throughout the MIE-4 and the pp150 amplicons. For the MIE-4 gene, 56 cloned PCR products were sequenced and 30 mutations (1 of 745 nucleotides) were observed. For the pp150 gene, 76 cloned PCR products were analysed, demonstrating a total of 20 (1 in 1,292 nucleotides) mutations.

DISCUSSION

In recent years it has been shown that latent HCMV is present in leukocytes [Stanier et al., 1989; Bevan et al., 1991; Taylor-Wiedeman et al., 1991, 1993] and in the arterial wall [Melnick et al., 1983; Yamashiroya et al., 1988; Hendrix et al., 1990, 1991] of healthy HCMV-seropositive individuals. In this report we demonstrate, by nested PCR, that HCMV is also present in the kidney, spleen, and liver of previously healthy trauma victims. We also present sequence analysis data which demonstrate a high sequence homology between the HCMV present in these organs and the reference strain AD169.

The data presented in this study confirm the observation that HCMV DNA is present in a variety of organs obtained from HCMV-seropositive individuals [Jahn et al., 1987; Naoumov et al., 1988; Arndt et al., 1990]. A wide tissue distribution of MCMV has been demonstrated in animal models during acute and latent infection [Myerson et al., 1984; Klotman et al., 1990; Collins et al., 1993; Stals et al., 1990].

Fidelity analysis of the procedures used demonstrated that 1 in 1,066 nucleotides for MIE-4 and 1 in 909 nucleotides for pp150 were misincorporated when starting from AD169 sequences. This means that for MIE-4 a total of 21 mutations and for pp150 a total of 28 mutations could be expected on the basis of the estimated fidelity and the total number of cloned PCR products sequenced. The total number of mutations observed for MIE-4 and pp150 (30 and 20, respectively) is consistent with the estimated fidelity, so it probably does not represent true mutations. The random distribution of these mutations further supports this conclusion. Other reports also demonstrate minor sequence variations within the MIE-4 gene [Zaia et al., 1990], but provide no fidelity data. None of the observed mutations produce stop signals as analysed by the PC gene program. The high degree of homology between the amplified fragments and the reference strain AD169 demonstrates that HCMV DNA is indeed detected, and the numerous negative controls exclude contamination with exogenous DNA.

The repeatedly observed mutations of the MIE-4 gene in the kidney of subject 5 and of the pp150 gene in the kidney and liver of subject 1 probably represent true mutations. As for patient 5, it seems that probably two types of the MIE-4 gene are present, and for patient 1 it seems that probably two types of the pp150 gene are detected. Whether these two types of genes represent two different HCMV strains, however, is not known. Only isolation and propagation of HCMV from these organs can answer this question. Excretion of more than one strain of virus has been demonstrated during active infection [Chou, 1989; Shen et al., 1993; Spector et al., 1984; Collier et al., 1989], but this phenomenon has up to now not been described during latency.

The presence of HCMV DNA in organs that are used for transplantation and in the spleen suggests that the spleen can be used to screen for HCMV DNA by PCR prior to transplantation. Seronegative organ recipients of organs containing two types of genes can then be carefully investigated for the emergence of HCMV strains carrying the same mutations.

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